American Journal of Hematology 52:90–95 (1996)

Generation of Antibodies to Heparin–PF4 Complexes Without Thrombocytopenia in Patients Treated With Unfractionated or Low-Molecular-Weight Heparin

Jean Amiral, Edith Peynaud-Debayle, Martine Wolf, Françoise Bridey, Anne-Marie Vissac, and Dominique Meyer

Serbio Research Laboratory, Gennevilliers Cedex (J.A., A-M.V.); Service d'Hématologie Biologique, Hôpital Antoine-Béclère, Clamart (E.P-D., M.W., F.B., D.M.), France

The incidence of antibodies to heparin–PF4 complexes (H–PF4) has been evaluated in patients who were under heparin therapy for more than 7 days: 109 patients treated with unfractionated heparin (UH) and 100 patients with low-molecular-weight heparin (LMWH). The presence of antibodies was identified in 17% of the former group and 8% of the latter. In both the UH and the LMWH groups, IgM antibodies were found in all but four patients who showed IgA antibodies. IgG isotypes were only detected in five patients and were consistently associated to either IgM or IgA antibodies. The follow-up of H–PF4 antibodies in 76 patients treated with UH from 1 to ≥12 days showed a relationship between the incidence of antibodies and the duration of therapy. Despite the presence of anti-H–PF4 antibodies there was no thrombocytopenia (<150 10^9/L) in the patients. A significant drop of platelets requiring the discontinuation of heparin was observed, however, in three patients, but their platelet count consistently remained >150 10^9/L. Our study demonstrates that the induction of antibodies to H–PF4 is a frequent phenomenon in patients treated with UH or with LMWH. The absence of thrombocytopenia and of clinical complications in these patients demonstrates that other conditions must be associated with H–PF4 antibodies for inducing type II HIT: optimal concentrations of heparin and PF4 in the blood circulation to allow the formation of macromolecular H–PF4 complexes, presence of activated platelets that present an increased binding of H–PF4 complexes, increased expression of FcγRIIA receptors, or presence of their H 131 phenotype. We conclude that the measurement of antibodies to H–PF4 complexes allows the detection of heparin-treated patients at risk of developing type II HIT.

Key words: heparin, UH, LMWH, HIT, anti-heparin–PF4 autoantibodies, platelet activation

INTRODUCTION

We have recently reported the frequent occurrence of antibodies to heparin–PF4 (H–PF4) complexes in patients with severe autoimmune heparin-induced thrombocytopenia (type II HIT) [1]. Generation of these antibodies was proposed as the cause of this clinical complication. This observation has been confirmed by other studies [2–6], although other target antigens may be involved in a few cases [5]. Based on our finding, a specific assay has been developed for testing for these heparin-dependent autoantibodies [1,6]. These antibodies are targeted to platelets by binding to the H–PF4 complexes, which are already fixed on the platelet surface through PF4 binding in a dose-dependent manner subsequent to a slight platelet activation by thrombin [7]. This antibody binding then induces platelet activation and aggregation, which can produce the clinical manifestations associated to this complication. In a retrospective kinetic study, we showed

Received for publication July 25, 1995; accepted January 17, 1996.

Address reprint requests to Dr. D. Meyer, Service d'Hématologie Biologique, Hôpital Antoine-Béclère, 157 rue de la Porte de Trivaux, 92141 Clamart, France.

Dr. Bridey is now at the Laboratoire d'Hématologie et d'Immunologie Biologiques, Hôpital Bichat, 46 rue Henri Huchard, 75018 Paris, France.

that antibodies were generated prior to the occurrence of thrombocytopenia and that their presence preceded the clinical complications [6]. Information is still lacking, however, on the true incidence of antibodies during heparin therapy and on their link to the occurrence of clinical symptoms such as thrombocytopenia and thrombosis. In 1993, Warkentin suggested that heparin-dependent antibodies might be induced in patients under heparin therapy, with a higher frequency than the occurrence of thrombocytopenia [8]. In order to determine the frequency of antibody generation during prolonged heparin therapy, antibodies to H–PF4 complexes were analyzed and isotyped in patients without thrombocytopenia receiving unfractionated heparin (UH) for more than 7 days and low-molecular-weight heparin (LMWH) for more than 10 days. We also investigated the development of antibodies to H–PF4 in patients treated with UH according to the duration of therapy. This report demonstrates that antibodies to H–PF4 complexes are observed in heparin-treated patients and that they are produced in a time-dependent manner. Most of the antibodies are of the IgM isotype; however, IgG and/or IgA may also be observed. Although these antibodies are not associated with severe thrombocytopenia, they must be considered alert signals. In association with other factors, they favour the occurrence of type II HIT.

PATIENTS, MATERIALS, AND METHODS

Laboratory Assays

Assay of anti-H–PF4 antibodies was performed as previously reported [1,6]. Briefly, H–PF4 complexes prepared by mixing 25 IU of heparin with 1 mg recombinant-PF4 (kindly provided by Dr. M. Poncz, Philadelphia) were covalently coupled on a covalink micro-enzyme-linked immunosorbent assay ELISA plate (Nunc, Roskilde, Denmark) at a 20-μg/ml concentration, then saturated with 2% goat serum. Plasmas were tested at a 1:50 dilution in a diluent containing 10% goal serum as previously reported [1]. Affinity-purified goat immunoglobulins specific for human IgG (γ specific), IgA (α specific) and IgM (μ specific) were obtained from Valbiotech (Paris, France). These immunoglobulins were coupled to horseradish peroxidase (HPO) (Sigma, St. Louis, MO) according to Nakane and Kawaoi [9] and were used for measuring the antibodies to H–PF4 complexes by ELISA as already described [6]. The three probes were pooled for the purpose of detecting all immunoglobulin isotypes reactive with H–PF4 complexes with a similar reactivity. Subsequently, IgG, IgA, or IgM antibody isotyping was performed on a positive specimen using the same ELISA technique while replacing the anti-IgG,A,M peroxidase conjugate by the specific anti-IgG-, anti-IgA-, or anti-IgM-peroxidase conjugates described above. Color development was obtained with an ortho-phenylenediamine/hydrogen peroxide substrate stopped with sulfuric acid. Absorbance was measured at 492 nm (A492). The negative range for the assay was defined by testing a control group including 63 healthy individuals and 30 patients with thrombocytopenia from other causes than HIT or autoimmune disorders. The upper limit of the normal range was then defined as the mean value +3 SD (i.e., 0.50). The global assay when performed at 20°C was then considered positive for A492 values above 0.50 and negative below 0.25 (the higher value obtained for the controls). A gray zone was defined for A492 values between 0.25 and 0.50. For the corresponding isotype analysis, the same approach identified positive values for A492 as values above 0.20. A HIT-positive plasma was used as control for checking this reactive range during each run. The assay was not affected by the presence of heparin concentrations in the tested plasma up to 2 IU/ml [6].

Blood Collection and Platelet Count

Blood was collected using either EDTA for platelet counting or 3.2% sodium citrate (9 vol blood per 1 vol of 0.109 M trisodium citrate). Following centrifugation of the blood samples (within 4 hr of collection) for 20 min at 3,000*g*, plasma supernatants were obtained and stored at −80°C. Samples were thawed for 15 min at 37°C just before use. Platelet counts were performed on the EDTA samples using a Coultronics instrument (Margency, France) and were compared to those before heparin therapy.

Patients

Two hundred eighty-five randomly selected hospitalized patients receiving a curative or prophylactic treatment with unfractionated heparin (UH) or low-molecular-weight heparin (LMWH) were tested for the presence of antibodies to H–PF4. These patients were hospitalized in various patient care units in our hospital. The main indications for curative treatment were deep vein thrombosis, pulmonary embolism, and myocardial infarction. Patients received either UH or LMWH according to the therapeutic protocol used by their attending physician. UH was given in continuous intravenous infusion, at a dose regimen of 3,000–10,000 international units (IU) per 24 hr. When LMWH was used, a total of 10,000–15,000 IU/day was given subcutaneously twice a day. Prophylactic treatments concerned heavy surgery (mainly orthopedic) and high-risk pregnancies with thrombotic antecedents. In this case, LMWH was given subcutaneously, once a day, at a dose regimen of 2,000–5,000 IU. Some of these patients could have had a previous exposure to heparin. In a first study, 109 patients, who were treated with UH [calciparin or heparin (Sanofi-Choay, Paris, France)] for more *than 7 days* (7–48), and 100 other patients treated with LMWH for 10 days were inves-

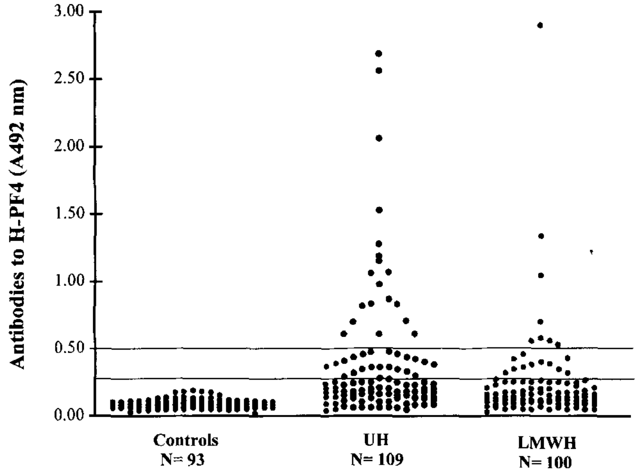

Fig. 1. Assay of antibodies to heparin–PF4 complexes (H–PF4) in controls (N = 93: 63 normal subjects and 30 patients with autoimmune diseases or thrombocytopenia, not treated with heparin), in 109 patients treated with unfractionated heparin (UH) for >7 days and in 100 patients treated with low-molecular-weight heparin (LMWH) for >10 days. A492 was consistently <0.25 in controls. A492 >0.50 was considered as positive. Patients with A492 between 0.25 and 0.50 were not considered positive.

tigated. The LMWH used were Fragmin (Kabi, Paris, France), Lovenox (Rhône Poulenc Rorer, Neuilly-sur-Seine, France), or Fraxiparine (Sanofi, Gentilly, France). For both groups, UH or LMWH therapy was the sole inclusion criterion considered. In a second study, 76 patients, treated with UH for variable time frames were tested at one of the following days: 1, 3, 5, 7–8, 9–11, ≥12. Seven of these patients had heparin therapy for 15–40 days. In addition to blood clotting assays and heparin measurements, patients were regularly tested for platelet count. Sixty-three normal individuals and 30 nonheparinized patients with thrombocytopenia or autoimmune diseases were used as controls.

RESULTS

Incidence of Antibodies to H–PF4 in Patients Treated With Heparin

The presence of antibodies to H–PF4 complexes was observed in 19 of the 109 patients (17%) treated with UH for more than 7 days and in 8 of the 100 patients (8%) treated with LMWH for more than 10 days (Fig. 1). Isotyping allowed confirmation of the presence of antibodies to H–PF4 complexes in these 27 patients. In the UH group, isotyping demonstrated that 12 of the 19 patients with antibodies to H–PF4 only developed IgM, whereas 3 patients developed IgA, and one IgM and IgA (Fig. 2a). IgG isotypes (A492 >0.20) were present in only 3 of these 19 patients and were associated in 2 cases with IgM and in one case with both IgA and IgM (Fig. 2a). In the LMWH group, isotyping of antibodies to H–PF4 in the 8 patients demonstrated the presence of IgM in 7 patients, IgA in one case, and a mixture of IgA and IgM in 2 patients. The IgG isotype was only observed in two cases, associated either with IgM (1 case) or with both IgA + IgM (1 case) (Fig. 2b). In addition to these 27 patients with clear evidence of antibodies to H–PF4, 21 patients among the UH group and 11 in the LMWH group were found in the gray zone (Fig. 1). As no patient with type II HIT has previously been found in the gray zone [6], these patients with questionable results were not considered positive. No severe thrombocytopenia (<150 10^9/L) was observed in any of the patients who developed antibodies (Table I). On the contrary, the mean platelet count was higher than at day 0 (Table I). A significant drop of platelets (36%, 40%, and 70% decrease, respectively, as compared to the initial platelet count) was observed, however, in 3 patients (2 with IgM and 1 with IgA isotypes), although their platelet count remained >150 10^9/L. This finding suggests that the latter patients could have developed clinical complications of type II HIT, and heparin was stopped on the day when the decrease of platelet count was observed.

Relationship Between Duration of Heparin Therapy and Development of Antibodies to H–PF4

In 76 patients treated with UH, there was a relationship between the development of antibodies to H–PF4 and the duration of therapy (Fig. 3). On day 1, 1 of 44 patients already showed positive results, and 2 others were questionable (in the gray zone), suggesting previous exposure to heparin [10,11]. On day 5, another patient became positive (A492 > 0.50) and 3 of 30 were in the gray zone. On days 7–8, 4 of 33 patients were positive and 6 were questionable, and at days ≥12, 8 of 33 patients were positive and ten others questionable. No patient required discontinuation of heparin because of thrombocytopenia. All the patients developed IgM isotypes, and about 20% showed IgG or IgA isotypes. IgG isotypes were already present on day 3 in one patient, whereas IgA isotypes were only observed on day 9 (Fig. 4). Eight patients under prolonged heparin therapy found to be negative for antibodies to H–PF4 on day 12 were still negative on days 20 (5 patients), 25 (2 patients), and 40 (1 patient). Only one patient who was in the gray zone on day 10 developed high levels of antibodies at day 20 (A492 = 2.56), and heparin was stopped.

DISCUSSION

This study demonstrates that despite the absence of thrombocytopenia, antibodies to H–PF4 complexes are

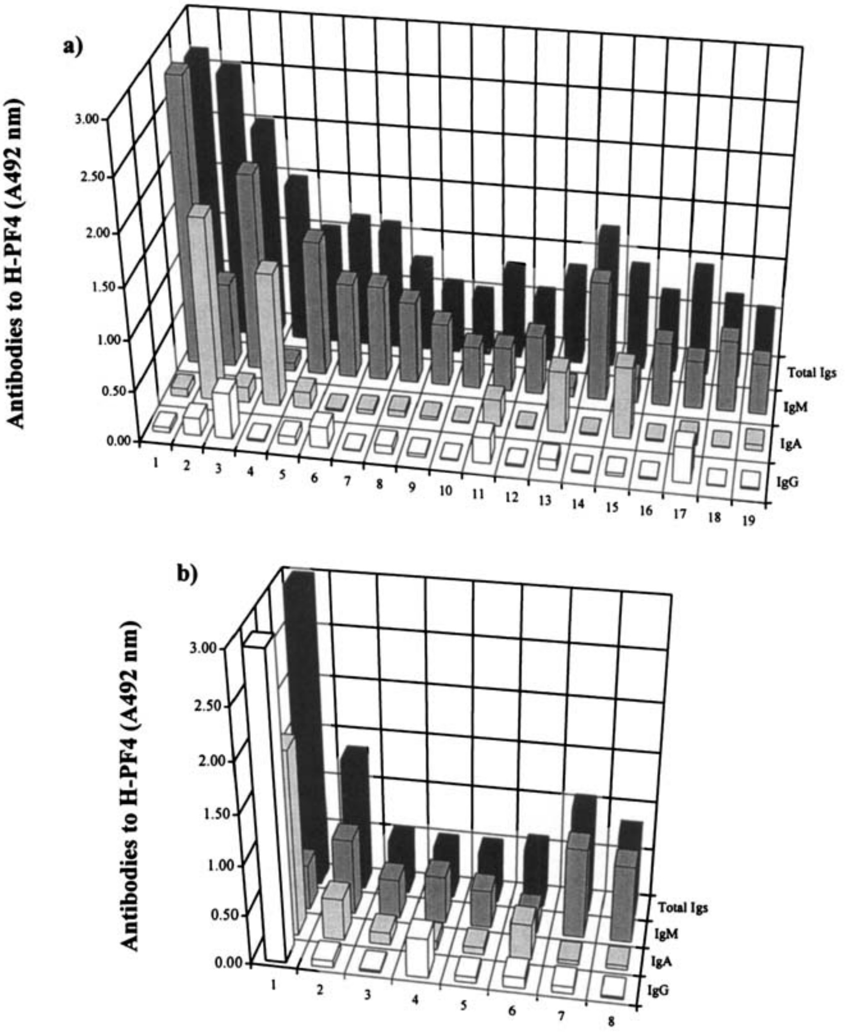

Fig. 2. Isotype distribution in patients with antibodies to heparin–PF4 complexes (H–PF4) treated with unfractionated heparin (UH) (N = 19) for more than 7 days (a) or with low-molecular-weight heparin (LMWH) (N = 8) for 10 days (b).

TABLE I. Presence of Antibodies to Heparin–PF4 Complexes in 109 Patients Treated With Unfractionated Heparin (UH) for More Than 7 Days or With Low-Molecular-Weight Heparin (LMWH) for 10 Days*

	UH				LMWH			
			Platelets 10^9/L				Platelets 10^9/L	
	N	A492 nm	Day 0	Day test	N	A492 nm	Day 0	Day test
Total	109	0.30 ± 0.30	291 ± 98	340 ± 122	100	0.24 ± 0.32	287 ± 148	351 ± 185
Negative	90	0.13 ± 0.05	278 ± 78	354 ± 126	92	0.13 ± 0.05	301 ± 164	345 ± 170
Positive	19	0.87 ± 0.31	290 ± 96	303 ± 115	8	1.01 ± 0.82	225 ± 50	442 ± 151

*Variations of A492 nm (mean ±SD) and platelet count (mean ±SD) in the overall groups (N = 109 for UH, N = 100 for LMWH) and in patients found negative or positive for the presence of antibodies to heparin–PF4 complexes.

frequently observed in patients under prolonged heparin therapy. The generation of antibodies increases with the duration of heparin (5–12 days), supporting the major interest of short-term heparin treatment and early initiation of oral anticoagulant treatment. These antibodies are present in both UH- and LMWH-treated groups, but their frequency is much lower in the LMWH (8% LMWH vs. 17% UH). The lower frequency in the LMWH group is consistent with the clinical observation and with the in vitro studies showing that LMWH is less reactive with platelets than UH [12]. Recently, in a prospective study of patients treated with heparin but without type II HIT,

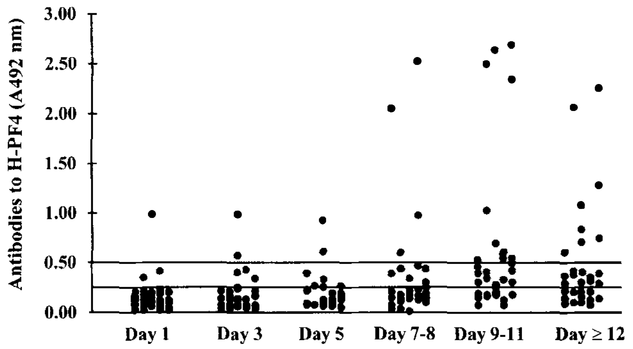

Fig. 3. Relationship between the duration of unfractionated heparin (UH) therapy and the incidence of antibodies to heparin–PF4 (H–PF4) in a follow-up study of 76 patients.

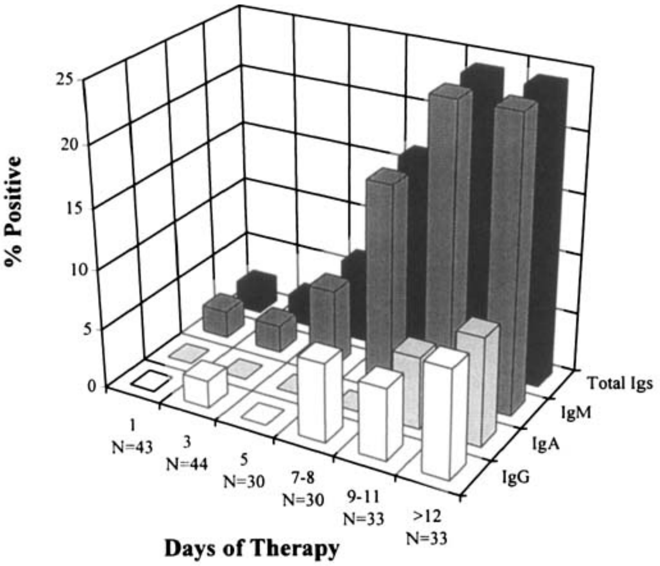

Fig. 4. Incidence of total antibody and isotype distribution in patients developing antibodies to heparin–PF4 (H–PF4), according to the duration of UH therapy, in a follow-up study of 76 patients.

Warkentin et al. [13] also showed that antibodies are more common with UH (7.8%) than with LMWH (2.2%). The lower incidence reported in the latter study may be related to a methodological problem, as the serotonin release assay used is less sensitive than the ELISA [5]. By contrast, Visentin recently reported a high frequency of antibodies to H–PF4 in 64% of patients with heart surgery [21]. This high frequency could result from the heavy clinical context of these patients, as well as from a previous exposure to heparin.

As heparin-dependent antibodies appear to require both the Fab and Fc fragments for inducing platelet activation through FcγRII receptors [14], the isotype distribution of antibodies and the presence of IgG in particular could be a key factor for the development of clinical complications [15–18]. This hypothesis was not fully confirmed however by our group. In a previous report [6], we showed that thrombocytopenia and thrombosis could occur in patients developing type II HIT associated to the presence of IgA or IgM isotypes or both IgA and IgM, but without IgG. Recently, we demonstrated that IgG isotypes were absent in about one-third of patients with type II HIT, and we reported that the incidence of thrombosis was similar to that in patients with IgG isotypes [22]. This observation suggests that platelet activation and aggregation can also be induced by IgM or IgA antibodies or by the association of both and that the presence of IgG isotypes is not necessarily required. We therefore proposed that PF4 binding to platelets play a major role in the development of type II HIT [6,22]. This PF4 binding occurs on the platelet surface subsequent to a slight thrombin activation; this has an important function in regulating platelet activation [7]. When macromolecular H–PF4 complexes are formed in the blood circulation, they bind to platelets through PF4. The binding of IgM and IgA antibodies to platelets would directly promote their activation. This mechanism appears unlikely, however, as it has only been suggested in one study [19] using $F(ab')_2$ fragments from monoclonal antibodies directed to an unidentified platelet surface antigen. Alternatively, platelet activation could be indirectly produced by the binding of antibodies to both platelets and other cells with receptors for antibodies: lymphocytes exposing FcμR receptors, monocytes and neutrophils exposing FcαR receptors.

The present study demonstrates that only 5 of 27 patients (18.5%) with H–PF4 antibodies in the absence of type II HIT develop IgG isotypes, whereas the presence of IgG isotypes was demonstrated in most (68.5%) of the patients with type II HIT [22]. The presence of H–PF4 antibodies in patients without clinical complications provides indirect evidence that other conditions are required for the development of type II HIT: (1) presence of optimal concentrations of heparin and PF4 in the blood circulation, allowing the formation of macromolecular H–PF4 complexes; (2) presence of activated platelets with enhanced H–PF4 complexes binding; and (3) influence of the FcγRIIA polymorphim as the frequency of the His 131 phenotype is increased in patients with HIT, suggesting that the latter phenotype is essential for IgG binding and platelet activation [23]. Such conditions only coexist in some patients treated with UH or LMWH, explaining why only a few of them demonstrate clinical complications of type II HIT. The observation of a significant platelet drop in three patients with anti-H–PF4 antibodies confirms our previous finding that antibodies precede the development of thrombocytopenia. Similarly, Hach-Wunderle et al. [20] demonstrated that platelet aggregation tests were positive in patients with heparin-associated thrombosis despite a normal platelet count. In

addition, as antibodies may react with PF4 complexed with heparin sulfate on endothelial cells [4], their binding could also produce endothelial activation and thus promote thrombosis [24]. We conclude that the measurement of antibodies to H–PF4 complexes is helpful for the monitoring of patients treated with UH or LMWH who are at increased risk of developing type II HIT.

ACKNOWLEDGMENTS

The authors thank C. Bahuon for her technical assistance, Dr. M. Poncz (Philadelphia, PA) for providing the construct, Dr. D. Kaczor for reviewing the English language, N. Hugon for producing recombinant PF4, and V. Penissard for expert typing and presentation of the manuscript.

REFERENCES

1. Amiral J, Bridey F, Dreyfus M, Vissac AM, Fressinaud E, Wolf M, Meyer D: Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin induced thrombocytopenia. Thromb Haemost 68:95, 1992.
2. Greinacher A, Pötzch B, Amiral J, Dummel V, Eichner A, Mueller-Eckhardt C: Heparin associated thrombocytopenia: Isolation of the antibody and characterization of a multimolecular pF4–Heparin complex as the major antigen. Thromb Haemost 71:247, 1994.
3. Kelton G, Smith JW, Warkentin TE, Hayward CPM, Denomme GA, Horsewood P: Immunoglobulin G from patients with heparin induced thrombocytopenia binds to a complex of heparin and platelet factor 4. Blood 83:3232, 1994.
4. Visentin GP, Ford SE, Scott JP, Aster RH: Antibodies from patients with heparin induced thrombocytopenia/thrombosis are specific for platelet factor 4 complexed with heparin or bound to endothelial cells. J Clin Invest 93:81, 1994.
5. Greinacher A, Amiral J, Dummel V, Vissac AM, Kiefel V, Mueller-Eckhardt C: Laboratory diagnosis of heparin associated thrombocytopenia and comparison of platelet aggregation test, heparin induced platelet activation test, and platelet factor 4/heparin enzyme linked immunosorbent assay. Transfusion 34:381, 1994.
6. Amiral J, Bridey F, Wolf M, Boyer-Neumann C, Fressinaud E, Vissac AM, Peynaud-Debayle E, Dreyfus M, Meyer D: Antibodies to macromolecular platelet factor 4 heparin complexes in heparin induced thrombocytopenia: A study of 44 cases. Thromb Haemost 73:21, 1995.
7. Capitanio AM, Niewiarowski S, Rucinski B, Tuzynski GP, Cierniewski CS, Hershock D, Kornecki E: Interaction of platelet factor 4 with human platelets. Biochim Biophys Acta 839:161, 1985.
8. Warkentin TE, Levine MN, Roberts RS, Gent M, Horsewood P, Kelton JG: Heparin induced thrombocytopenia is more common with unfractionated heparin than with low molecular weight heparin. Thromb Haemost 69:911, 1993.
9. Nakane PK, Kawaoi A: Peroxidase labeled antibody: A new method of conjugation. J Histochem Cytochem 22:1084, 1974.
10. Laster J, Elfrink R, Silver D: Reexposure to heparin of patients with heparin associated antibodies. J Vasc Surg 9:677, 1989.
11. Cadroy Y, Amiral J, Raynaud H, Brunel P, Mazaleyrat A, Sauer M, Sié P: Evolution of antibodies anti-PF4/heparin in a patient with a history of heparin induced thrombocytopenia reexposed to heparin. Thromb Haemost 72:782, 1994.
12. Brace LD, Fareed J: Heparin-induced platelet aggregation: Dose/response relationships for a low molecular weight heparin derivative (PK 10169) and its subfractions. Thromb Res 42:769, 1986.
13. Warkentin TE, Levine MN, Hirsh J, Horsewood P, Roberts RS, Gent M, Kelton JG: Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. N Engl J Med 332:1330, 1995.
14. Kelton JG, Sheridan D, Santos A, Smith J, Steeves K, Smith C, Brown C, Murphy WG: Heparin induced thrombocytopenia: Laboratory studies. Blood 72:925, 1988.
15. Laroche J, Clofent-Sanchez G, Jallu V, Hourdillé P, Vezon G, Nurden AT: Further evidence that heparin dependent thrombocytopenia may result from Fc receptor-mediated interactions. Nouv Rev Fr Hematol 34:111, 1992.
16. Chong BH, Pilgrim RL, Cooley MA, Chesterman CN: Increased expression of platelet IgG Fc receptors in immune heparin induced thrombocytopenia. Blood 81:988, 1993.
17. Chong BH: Heparin induced thrombocytopenia. Br J Haematol 89: 431, 1995.
18. Warkentin TE, Hayward CPM, Boshkov LK, Santos AV, Sheppard JAI, Bode AP, Kelton JG: Sera from patients with heparin induced thrombocytopenia generate platelet derived microparticles with procoagulant activity: An explanation for the thrombotic complications of heparin induced thrombocytopenia. Blood 84:3691, 1994.
19. De Reys S, Hoylaerts MF, De Ley M, Vermylen J, Deckmyn H: Fc-independent cross-linking of a novel platelet membrane protein by a monoclonal antibody causes platelet activation. Blood 84:547, 1994.
20. Hach-Wunderle V, Kainer K, Krug B, Müller-Berghaus G, Pötzsch B: Heparin-associated thrombosis despite normal platelet counts. Lancet 344:469, 1994.
21. Visentin GP, Malik MI, Menden K, Aster RH: A prospective study of the formation of antibodies reactive with heparin: PF4 complexes in patients treated with heparin. Blood 84:80a, 1994.
22. Amiral J, Wolf M, Fischer AM, Boyer-Neumann C, Vissac AM, Meyer D: Pathogenicity of IgA and/or IgM antibodies to heparin–PF4 complexes in patients with heparin induced thrombocytopenia. Br J Haematol (in press).
23. Brandt JT, Osborne JM, Chacko G, Anderson CL: The role of FcγRIIA phenotype in heparin-induced thrombocytopenia. Thromb Haemost 73: 1064, 1995.
24. Cines DB, Tomaski A, Tannenbaum S: Immune endothelial-cell injury in heparin-associated thrombocytopenia. N Engl J Med 316:581, 1987.